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Anne-Marie PINEL et al.

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For: NOVEL PEPTIDIC CONJUGATES FOR ALOPECIA PREVENTIVE AND CURATIVE TREATMENT Examiner: J. HA

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Michel Hocquaux, declare the following:

I am a co-inventor of the above-identified U.S. Patent application.

I have read and understand the specification and claims to the above-identified application and the outstanding Office Action of June 28, 2007.

I am experienced in the field of cosmetics, as demonstrated by my *curriculum viteae*, a copy of which is attached hereto as Exhibit A.

I understand the Examiner's rejections as set forth in the Office Action of June 28, 2007,

including rejections based on enablement under 35 U.S.C. § 112, first paragraph. As evidence of enablement of the presently claimed invention, I submit hereinbelow additional empirical and objective evidence of the efficacy of the presently claimed invention. The experiments presented below were conducted by Bioalternatives and located in Gençay (France) under my supervision and direction. These experiments provide data which demonstrate the effectiveness of the presently claimed methods and compositions for the purposes recited in the present claims.

Briefly, the experiments were performed as follows, generating the following data:

The *in vitro* growth of human hair follicles in a control medium or in presence of peptide conjugates claimed in the present application has been analyzed as follows.

METHODS

Preparation of human hair follicles

Human hair follicles were obtained from human scalps obtained after plastic surgery and their in vitro growth analyzed as described in Philpott et al., *J Cell Sci.*, 1990, 97(Pt 3):463-71 (copy of which is attached hereto as Exhibit B). Briefly, human anagen hair follicles were isolated by microdissection from human scalp skin obtained after plastic surgery.

Isolation of the hair follicles was achieved by cutting the follicle at the dermo-subcutaneous fat interface using a scalpel blade. Intact hair follicles were then removed from the fat using watchmakers' forceps.

Isolated hair follicles were then maintained free-floating in 500 µL of Williams E medium supplemented with 1 % fetal calf serum, 1 mM-L-glutamine, insulin (10 µg/ml), transferring (10 µg/ml), hydrocortisone (10 ng/ml), sodium selenite (10 ng/ml), Fungizone (2.5

µg/ml), penicillin/streptomycin (100 units/ml) and trace elements (Gibco) in individual wells of 24-well multi-well plates. Various concentrations of several peptide conjugates according to the presently claimed invention were also added to the medium. The following peptides were added to supplemented Williams E medium:

Name	Formula	Corresponds to
FT04	Ac-Arg-Lys-Asp-Val-DHomoPhe	Claim 4, peptide conjugate number 1, wherein A = acetic acid
RE20	Ac-DLys-Asp-Val-Dopa-NH_2	Claim 4, peptide conjugate number 4, wherein A = acetic acid

In negative control experiments, no additional compound was added to the supplemented Williams E medium.

Culture of follicles and measurement of growth

Follicles were maintained free-floating in individual wells of 24-well multi-well plates, at 37 °C in an atmosphere of 5% CO_2/95 % air for 7 (first series) or 8 (second series) days. This permitted detailed measurements to be made on the length of individual hair follicles. Measurements were made on day 7 or 8 using a Nikon Diaphot inverted binocular microscope with eye-piece measuring graticule.

RESULTS

The mean supplementary percentage of growth of hair follicles in the presence of FT04 or RE20 compared to hair follicles in control medium was calculated. Results are displayed in following Table 1:

Compound added	Concentration	Mean supplementary growth % compared to negative control
FT04	$1.4\ 10^{-8}$ M	46%
RE20	10^{-6} M	33%

For each peptide conjugate, the experiment was conducted on at least 12 human hair follicles.

CONCLUSION

These results clearly show that the peptide conjugates, as exemplified by peptide conjugates FT04, RE20, trigger an increase in the growth of human hair.

STATEMENT UNDER 18 U.S.C. § 1001

I hereby declare that all statements made herein of any own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 26.11.07

Dr. Michel Hocquaux

Attachments: Exhibit A - *curriculum viteae* of Dr. Michel Hocquaux
Exhibit B – copy of Philpott et al., *J Cell Sci.*, 1990, 97(Pt 3):463-71

Human hair growth *in vitro*

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Summary

We report for the first time the successful maintenance and growth of human hair follicles *in vitro*. Human anagen hair follicles were isolated by microdissection from human scalp skin. Isolation of the hair follicles was achieved by cutting the follicle at the dermo–subcutaneous fat interface using a scalpel blade. Intact hair follicles were then removed from the fat using watchmakers' forceps.

Isolated hair follicles maintained free-floating in supplemented Williams E medium in individual wells of 24-well multiwell plates showed a significant increase in length over 4 days. The increase in length was seen to be attributed to the production of a keratinised hair shaft, and was not associated with the loss of hair follicle morphology. [*methyl*-^{3}H]thymidine autoradiography confirmed that *in vitro* the *in vivo* pattern of DNA synthesis was maintained; furthermore, [^{35}S]methionine labelling of keratins showed that their patterns of synthesis did not change with maintenance.

The importance of this model to hair follicle biology is further demonstrated by the observations that TGF-β1 has a negative growth-regulatory effect on hair follicles *in vitro* and that EGF mimics the *in vivo* depilatory effects that have been reported in sheep and mice.

Key words: human hair follicles, organ maintenance, growth factors.

Introduction

The hair follicle is composed of epithelial components (the matrix and outer root sheath) and dermal components (the dermal papilla and connective tissue sheath). Hair growth, which is effected by the division of the hair follicle matrix cells under control of the dermal papilla, is cyclical in the mammal. Three distinct stages of hair growth can be identified, an active phase (anagen) during which hair growth occurs, an intermediate regressive (catagen) stage and a resting phase (telogen) during which no cell proliferation occurs. The factors that regulate cell division within the hair follicle matrix cells, and which control the hair growth cycle, are poorly understood, although growth factors (Moore *et al.* 1981; Green *et al.* 1983; Green and Couchman, 1984; Panaretto *et al.* 1984; Nanney *et al.* 1984; Akhurst *et al.* 1988; Green, 1989; Messenger, 1989), steroid hormones (Takayasu and Adachi, 1972; Schweikert and Wilson, 1974; Sultan *et al.* 1989), dermo–epithelial interactions (Jahoda *et al.* 1984) and the immune system (Sawada *et al.* 1987; Pauss *et al.* 1989) have been implicated. Our lack of understanding of the regulation of hair growth has been caused in part, by the lack of good *in vitro* models (Philpott *et al.* 1989; Buhl *et al.* 1989). We now report for the first time on the successful growth of human hair *in vitro*, and on the *in vitro* effects of growth factors and mitogens on our model. In particular, we show that epidermal growth factor (EGF) mimics the *in vivo* depilatory action of EGF, resulting in the formation of a club hair-like structure; and that transforming growth factor beta one (TGF-β1) may serve as a negative growth regulatory factor for the hair follicle.

Materials and methods

Materials

Williams E medium (minus glutamine), L-glutamine, Fungizone, penicillin and streptomycin were supplied by Gibco, all other tissue culture supplements came from Sigma. Polycarbonate filters were supplied by the Nucleopore corporation. All radiochemicals were from Amersham, GF/C filters came from Whatman and ATP monitoring kits were supplied by LKB Instruments Ltd. Mouse EGF and TPA were purchased from Sigma. Porcine TGF-β1 was from R&D systems Inc; synthetic human IGF-1 was supplied by Bachem Feinchemikalien. Minoxidil was a kind gift from Unilever Research, Colworth House, Sharnbrook, Bedford; EGF, TGF-β1 and IGF-1 were all assayed for mitogenic activity using 3T3 or keratinocyte test cells at Unilever and were found to have the expected biological activities.

Isolation and maintenance of human hair follicles

Human anagen hair follicles were isolated by microdissection from human scalp skin, taken from females aged 35–55 undergoing facelift surgery. Isolation of hair follicles was achieved by using a scalpel blade to cut through the skin at the dermo–subcutaneous fat interface. The intact hair follicle bulb was removed from the subcutaneous fat, under a stereo dissecting microscope, using watchmakers' forceps, by gently gripping the outer root sheath of the follicle in the forceps and pulling the hair follicle from the subcutaneous fat. This results in the isolation of intact hair follicle bulbs without sustaining any visible damage, a factor that is essential if successful maintenance of hair follicles is to be achieved.

Isolated hair follicles were maintained in 500 μl of Williams E medium with supplements as previously described (Philpott *et al.*

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1989). Follicles were maintained free-floating in individual wells of 24-well multi-well plates, at 37°C in an atmosphere of 5% CO_2/95% air. This permitted detailed measurements to be made on the length of individual hair follicles. Measurements were made using a Nikon Diaphot inverted binocular microscope with eye-piece measuring graticule.

Rates of DNA and protein synthesis

The rates of DNA and protein synthesis in isolated hair follicles were investigated by measuring the rates of incorporation of [*methyl*-^{3}H]thymidine and [U-^{14}C]leucine, respectively, into perchloric acid (PCA)-precipitable material. Incubations were carried out in plastic Eppendorf tubes containing 500 μl of Williams E medium supplemented with 1 μCi of 3 μM [*methyl*-^{3}H]thymidine (specific activity 0.67 mCi μmol^{-1}) and 0.5 μCi of 0.5 mM [U-^{14}C]leucine (specific activity 2 mCi mmol^{-1}); samples of thymidine and leucine were freeze dried prior to the addition of the Williams E medium to remove all traces of ethanol. Eppendorf tubes containing hair follicles were then incubated in stoppered plastic tubes containing 0.5 ml of distilled water in a gently shaking water bath at 37°C in an atmosphere of 5% CO_2/95% O_2. Incubations were carried out for 3 h.

After incubations were complete, the Eppendorf tubes containing the hair follicles were removed from their plastic tubes and briefly centrifuged at 12 000 *g* to bring down the hair follicles. The supernatant was then removed with a Pasteur pipette taking care not to remove the hair follicles as well. The follicles were then washed by resuspending them in 1 ml of PBS supplemented with 10 mM thymidine and 10 mM leucine; the follicles were then briefly centrifuged as before and the supernatant removed. After three such washes the follicles were resuspended in 1 ml of 0.1 M EDTA, pH 12.3, and transferred using a Pasteur pipette to a ground-glass homogeniser. Following homogenisation, the homogenate was transferred to an Eppendorf tube and centrifuged for 15 min at 12 000 *g*, to precipitate cell debris, after which the supernatant was removed for assay. Samples (100 μl) were removed for total DNA assay, and macromolecules in the remaining supernatant were then precipitated by the addition of 500 μl of 25% (v/v) PCA. The samples were then left overnight at 4°C.

The resulting precipitate was collected onto Whatman GF/C filters, under vacuum. The filters were then washed with 10 ml of 10% (w/v) trichloroacetic acid (TCA) followed by 5 ml of 5% (w/v) TCA and then dried with 1 ml of ethanol/diethyl ether (1:1, v/v). Radioactivity was counted in 10 ml of Optifluor® scintillant using dual counting liquid scintillation spectrometry. Control experiments were carried out in which the cell debris was solubilised in 1 ml of Soluene® and radioactivity counted in 10 ml of Optifluor® as described above, these controls showed that less than 10% of incorporated radioactivity was discarded in the cell debris pellet.

Hair follicle DNA content

Total hair follicle DNA content was determined using the fluorometric diaminobenzoic acid method (DABA) of Fiszer-Szafarz *et al.* (1981). Portions (100 μl) of sample were freeze dried under vacuum using a Virtis unitrap vacuum freeze drier. DABA (0.45 g ml^{-1}) was made up in distilled water decolorised by shaking with activated charcoal (0.15 g ml^{-1}) for 3 h, then filtered through a Millipore filter (0.45 μm) and stored in a glass vial in the dark until required (DABA was prepared fresh daily and not stored overnight). DABA (100 μl) was then added to the samples, which were incubated in a water bath for 45 min at 60°C. After this 1.5 ml of 1 M HCl was added to the samples and the fluorescence measured using a Perkin Elmer LS5 spectrofluorometer with an excitation wavelength of 420 nm and an emission wavelength of 520 nm. DNA standards were made up in 0.1 M EDTA and treated in exactly the same way as the samples.

Hair follicle ATP contents

This was measured using LKB-ATP monitoring kits based on the luciferin luciferase assay of ATP (Stanley and Williams, 1969). Hair follicles were placed in 500 μl of Williams E medium to which was added 100 μl of 20% PCA; the follicles were then left on ice for 30 min, following which 20 μl of sample was removed and neutralised with KOH. The sample was then centrifuged at 12 000 *g* for 5 min, after which a 10 μl sample was taken and assayed for ATP by adding 80 μl of 0.1 M Tris–acetate buffer, pH 7.75, followed by 10 μl of monitoring reagent. The ATP content was then measured using a LKB 1250 luminometer.

Autoradiography

Hair follicles were incubated for 6 h in 500 μl Williams E medium containing 5 μCi [*methyl*-^{3}H]thymidine (spec. act. 3.3 μCi nmol^{-1}). After incubation follicles were washed in PBS supplemented with 10 mM thymidine and then fixed for 1 h in phosphate-buffered formaldehyde. Follicles were then mounted in 3% agar fixed overnight in phosphate-buffered formaldehyde and then embedded in wax and sectioned. Autoradiographs were prepared using Ilford K5 dipping emulsion. Sections were stained using 0.1% Toluidine Blue.

Patterns of keratin synthesis

These were investigated by incubating the hair follicles in 500 μl Williams E medium containing 100 μCi of 1 mM [^{35}S]methionine (spec. act. 0.22 μCi nmol^{-1}) for 24 h at 37°C. Follicles were then washed three times in PBS containing 10 mM methionine and then homogenised in ice-cold lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM Tris–HCl, 30 mM sodium pyrophosphate, pH 7.4; Green *et al.* 1986). The homogenate was centrifuged at 12 000 *g* in an Eppendorf microtube for 15 min and the supernatant was discarded. The pellet was then twice extracted with a high-salt buffer (600 mM KCl, 5 mM EDTA, 5 mM EGTA, 50 mM Tris–HCl, pH 7.4; Mischke and Wilde, 1987). The supernatant was discarded and the insoluble pellet analysed by sodium dodecyl sulphate (SDS)–acrylamide gel electrophoresis as described by Laemlii (1970). Gels were then dried under vacuum and autoradiographs produced using Kodak X-OMAT diagnostic film.

Immunoblot analysis of proteins to identify keratins was carried out by electrophoretic transfer from gels to nitrocellulose membranes (Towbin *et al.* 1979), using a Bio-Rad Trans Blot system (Bio-Rad Laboratories). Membranes were incubated for 2 h at room temperature in PBS containing 5% skimmed milk, 0.05% Tween 20 to reduce non-specific binding, and then overnight with a wide spectrum rabbit anti-keratin polyclonal antibody (Dakopatts). The membrane was then washed for 30 min in PBS containing 0.05% Triton X-100, and then incubated for 2 h with a 1:1000 dilution of goat anti-rabbit IgG peroxidase conjugate (Sigma). Bands were visualised by incubating the membranes with PBS containing 0.5 mg ml^{-1} 3,3'-diaminobenzidine, 0.01% H_2O_2.

Results

Isolation and maintenance of hair follicles

It was found that by cutting human skin into thin strips approximately 3–5 mm×10 mm and then using a scalpel blade to cut away the subcutaneous fat at the level of the sebaceous gland, and using a pair of watchmakers' forceps, it was possible to isolate in excess of 100 human anagen hair follicles in 1–2 h from a piece of skin 4 cm×2 cm. Hair follicles in the early catagen stage of their growth cycle were occasionally seen, but were not used in these experiments.

Measurements made on freshly isolated human hair follicles and at 24 h intervals show (Fig. 1) that *in vitro* isolated human hair follicles significantly increased in length over 4 days in culture ($P<0.001$); the rate of growth *in vitro* being 0.3 mm a day ($n=6$ patients, 36 follicles in total), which approximates closely to that seen *in vivo* (Myers and Hamilton. 1951).

Photographs taken of freshly isolated and maintained

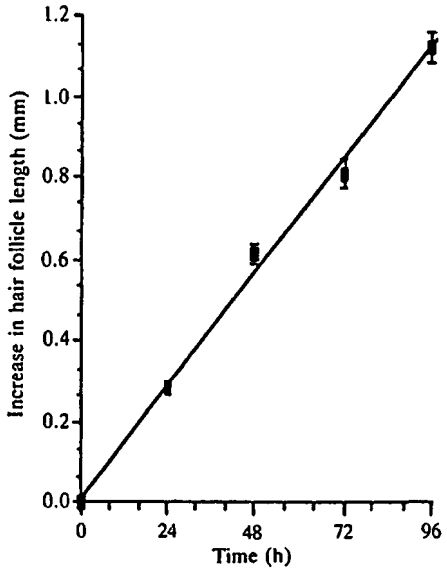

Fig. 1. Human hair follicle growth *in vitro*. Graph shows human hair follicle growth in culture over 96 h. Results expressed as the mean±S.E.M. for sequential measurements made on hair follicles isolated from $n=6$ skin biopsies (minimum of 6 hair follicles used from each biopsy).

hair follicles (Fig. 2) show that the increase in length over 4 days was not associated with any disruption of hair follicle architecture. In particular, the length increase can be seen from Fig. 2 to be attributed to the production of a keratinised hair shaft.

In order to determine whether the increase in hair follicle length observed above was due to the normal mechanisms of cell proliferation and migration, [*methyl*-^{3}H]thymidine autoradiography was carried out. Fig. 3A shows that in the freshly isolated follicles the typical pattern of DNA synthesis is taking place, with the majority of thymidine uptake occurring in the matrix cells of the hair follicle bulb, adjacent to the dermal papilla. Autoradiography carried out on hair follicles maintained for 4 days (Fig. 3B) shows that over 4 days of maintenance this pattern remains constant.

The pattern of keratin synthesis was studied by incubating hair follicles with [^{35}S]methionine for 24 h at 37 °C, after which cell extracts were separated by one-dimensional SDS–PAGE and analysed by autoradiography. The pattern of keratin synthesis observed in freshly isolated hair follicles under our conditions is shown in Fig. 4A (lane 1). In freshly isolated hair follicles using a 10 % gel we were able to resolve five major bands; a doublet of 56 and 59K (K=$10^3 M_r$) and a triplet of 48/49/50K. We also observed a number of faint lower molecular weight bands between 40 and 46K, which included a doublet at 44/46K. Control experiments using immunoblot analysis of gels with a wide-spectrum keratin antibody confirmed that all these bands were keratins (Fig. 4B).

It was also observed that the pattern of keratin synthesis remained unchanged in hair follicles maintained for 4 days (Fig. 4A, lane 2).

The effects of growth factors and mitogens on human hair follicle growth in vitro *and on the rates of* [methyl-^{3}*H]thymidine and [U-*^{14}*C]leucine uptake and on hair follicle ATP content*

The results of this study are shown in Table 1. All measurements of hair follicle length were carried out over a 72 h period and rates of [*methyl*-^{3}H]thymidine and [U-^{14}C]leucine uptake and hair follicle ATP content were measured after 72 h in culture.

Table 1 shows that for hair follicles maintained for 72 h in Williams E medium containing 1 % FCS the rate of hair follicle growth was 0.81±0.04 mm per 72 h, the rate of [*methyl*-^{3}H]thymidine uptake was 2.57±0.35 pmol μg^{-1} DNA per 3 h (mean±S.E.M.), and the rate of [U-^{14}C]leucine uptake 204±24 pmol μg^{-1} DNA per 3 h (mean±S.E.M.); hair follicle ATP content was 713±65 pmol follicle^{-1} (mean±S.E.M.).

When hair follicles were maintained with EGF (10 ng ml^{-1}) the rate of hair follicle growth was 0.70±0.05 mm per 72 h and was not significantly different from that of follicles maintained in 1% FCS alone, but there was a most striking change in the morphology of hair follicles maintained with 10 ng ml^{-1} EGF. Fig. 5 shows sequentially at 24 h intervals the changes that occur in the overall morphology of human hair follicles maintained with EGF (10 ng ml^{-1}). These observations

Table 1. *The effects of growth factors on* in vitro *hair follicle growth,* [methyl-^{3}*H]thymidine and [U-*^{14}*C]leucine uptake and hair follicle ATP content*

Treatment	Follicle growth (mm over 72 h) (n=6 samples)	[^{3}H]thymidine (pmol μg^{-1} DNA per 3 h) (n=6 samples)	[^{14}C]leucine (pmol μg^{-1} DNA per 3 h) (n=6 samples)	ATP content (pmol follicle^{-1}) (n=3 samples)
1 % FCS	0.81±0.04	2 57±0 35	204±24	713±65
TGF-β1 (10 ng ml^{-1})	0.57±0.03***	1.56±0.21*	175±22	653±20
IGF-1 (30 ng ml^{-1})	0.76±0.05	4 04±0 39*	255±30	485±39*
EGF (10 ng ml^{-1})	0.70±0.05	1.19±0 37*	357±71	698±43
TPA (100 ng ml^{-1})	0.46±0 04***	1.29±0.29*	165±28	297±16*
20 % FCS	0.85±0.05	2.64±0.37	328±42	Not measured
Serum-free	0.93±0.03*	2.71±0.64	193±28	610±54
Minoxidil				
200 μg ml^{-1}	0.63±0.05*	1.78±0.27	169±21	694±28
10 μg ml^{-1}	0.83±0.03	3.51±0.62	232±14	630±52
200 ng ml^{-1}	0.91±0.03	2.47±0.70	148±43	618±98

Hair follicles were isolated and maintained in Williams E medium containing supplements as described in the text, with the additional growth-regulatory factors as listed in the table. Hair follicle measurements were made on at least 6 hair follicles from each sample of skin. Rates of [*methyl*-^{3}H]thymidine and [U-^{14}C]leucine uptake were measured after the hair follicles had been maintained for 72 h in the presence of the relevant growth factors. Experiments were carried out using 5 follicles in duplicate from each skin sample. Hair follicle ATP contents likewise were measured after hair follicles had been maintained for 72 h with the relevant growth factor using 4 follicles in duplicate from each sample. Statistical analysis was carried out using Student's t-test to compare differences between follicles maintained with 1 % FCS and treated follicles (*P<0.05, **P<0 01, ***P<0.001).

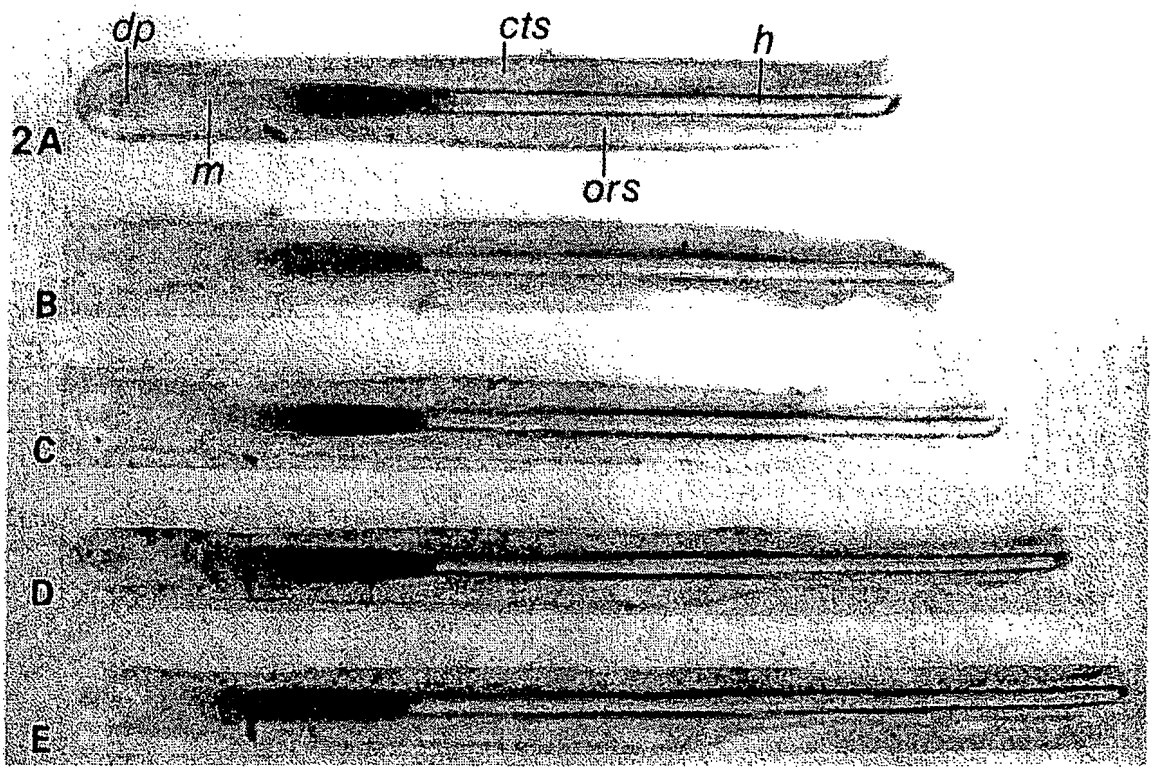

Fig. 2. Light micrographs taken under an inverted microscope showing the sequential growth of the same hair follicle in culture over 96 h. Clearly visible are the dermal papilla (dp), hair follicle matrix (m), outer root sheath (ors), connective tissue sheath (cts) and hair shaft (h). The increase in hair follicle length can be seen to be attributed to the production of a hair shaft. (A) Freshly isolated hair follicle; (B) after 24 h maintenance; (C) 48 h; (D) 72 h, and (E) 96 h.

show that over a 72 h period, human hair follicles maintained with 10 ng ml^{-1} EGF show considerable changes in their hair follicle morphology, especially in the hair follicle bulb where the hair shaft forms a club hair-like structure. This structure then moves slowly upwards within the hair follicle over 72 h until after 5 days it is nearly extruded from the hair follicle. There was moreover a significant decrease in the rate of [*methyl*-^{3}H]thymidine uptake to 1.19±0.37 pmol μg^{-1} DNA per 3 h (mean± S.E.M.) when compared with that for follicles maintained in 1% FCS, the rate of [U-^{14}C]leucine uptake at 357±71 pmol μg^{-1} DNA per 3 h, and a hair follicle ATP content of 698±32 pmol follicle^{-1} were, however, not significantly different.

In the presence of TGF-β1 the rate of increase of hair follicle length was found to be 0.57±0.03 mm per 72 h (mean±S.E.M.); this was significantly less (P<0.001) than that of hair follicles maintained with 1% FCS. The rate of [*methyl*-^{3}H]thymidine uptake for hair follicles maintained with TGF-β1 was 1.56±0.21 pmol μg^{-1} DNA per 3 h (mean±S.E.M.), which was also significantly less than that of follicles maintained with 1% FCS (P<0.05). The rate of [U-^{14}C]leucine uptake at 175±22 pmol μg^{-1} DNA per 3 h was not, however, significantly different, and neither was the hair follicle ATP content at 653±20 pmol follicle^{-1}.

IGF-1 (30 ng ml^{-1}) had no significant effect on hair follicle length *in vitro*, but did significantly increase (P<0.05) the rate of [*methyl*-^{3}H]thymidine uptake to 4.04±0.39 pmol μg^{-1} DNA per 3 h (mean±S.E.M.). IGF-1 did not, however, have any significant effect on the rates of [U-^{14}C]leucine uptake, which was 255±30 pmol μg^{-1} DNA per 3 h (mean±S.E.M.), but did significantly reduce the ATP content of the hair follicle to 485±39 pmol follicle^{-1} (mean±S.E.M., P<0.05).

For hair follicles maintained with 12-*O*-tetra-decanoyl-phorbol-13-acetate (TPA, 100 ng ml^{-1}) the rate of growth was reduced to 0.46±0.04 mm per 72 h; this value was significantly less (P<0.001) than that of hair follicles maintained with 1% FCS alone. The rate of [*methyl*-^{3}H]thymidine uptake for hair follicles was 1.29±0.29 pmol μg^{-1} DNA per 3 h (mean±S.E.M.) and this value too was significantly less than that for hair follicles maintained in 1% FCS alone (P<0.05). TPA had no significant effect on the rate of [U-^{14}C]leucine uptake, which was 165±28 pmol μg^{-1} DNA per 3 h (mean±S.E.M.), but did significantly reduce the hair follicle ATP content to 297±16 pmol follicle^{-1}.

For hair follicles maintained in serum-free Williams E medium the rate of hair follicle growth was 0.93±0.03 mm per 72 h (mean±S.E.M.), this value was significantly higher than that for follicles maintained in Williams E medium containing 1% FCS (P<0.05). There was no significant difference between the rates of [*methyl*-^{3}H]thymidine or [U-^{14}C]leucine uptake in follicles maintained in serum-free medium when compared with those maintained in medium containing 1% FCS, nor was there any significant difference between the ATP contents of hair follicles maintained in serum-free medium or 1% FCS. For hair follicles maintained in 20% FCS there was also no significant difference in the rate of hair follicle elongation and the rates of [*methyl*-^{3}H]thymidine uptake and [U-^{14}C]leucine uptake or hair follicle ATP contents.

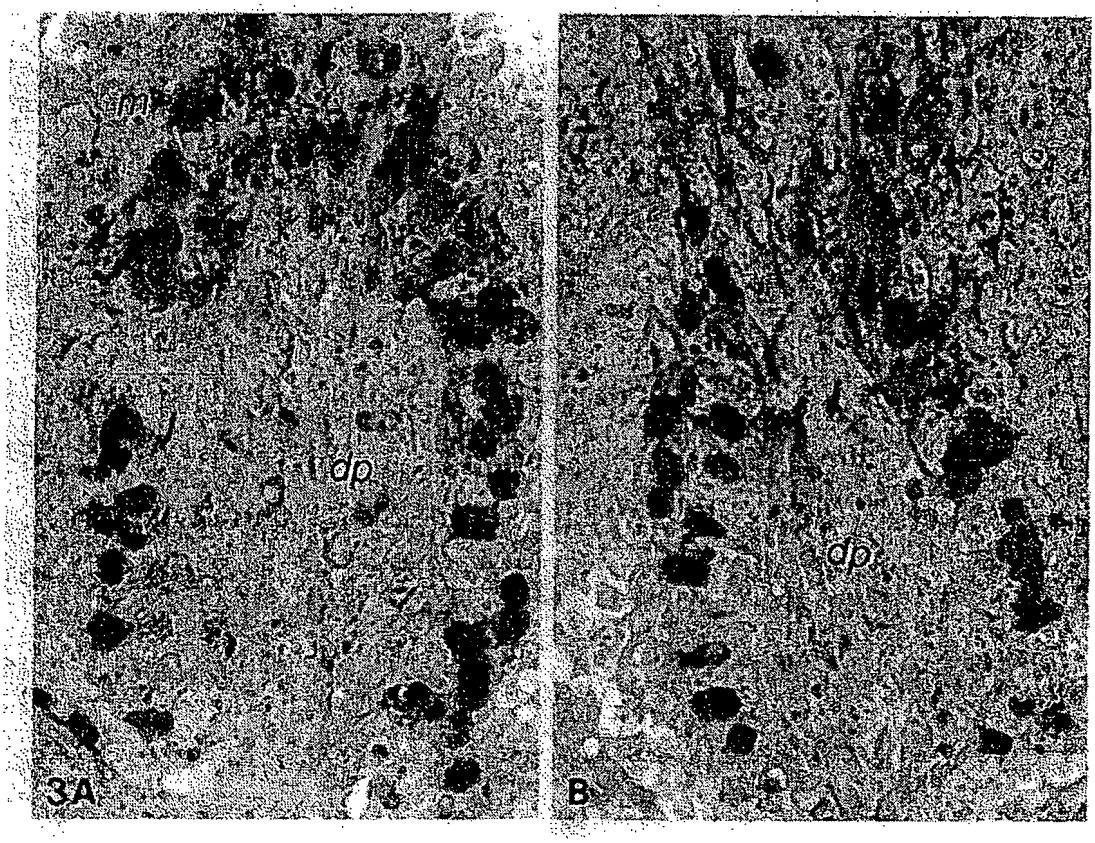

Fig. 3. Tritiated thymidine autoradiographs of isolated human hair follicles showing: (A) freshly isolated; and (B) 96 h maintained follicles. Freshly isolated hair follicles show the typical pattern of DNA synthesis in the hair follicle with the majority of thymidine uptake occurring in the matrix cells (m) of the hair follicle bulb, adjacent to the dermal papilla (dp). After 96 h in culture the pattern of DNA synthesis remains unchanged.

Experiments carried out on hair follicles maintained with Minoxidil at 200 ng ml^{-1} or at 10 μg ml^{-1} showed that neither of these had any significant effect on either the rates of hair follicle growth over 72 h or on the rates of [*methyl*-^{3}H]thymidine uptake or [U-^{14}C]leucine uptake, nor was there any significant effect on hair follicle ATP content. For hair follicles maintained with Minoxidil at 200 μg ml^{-1} for 72 h there was a significant inhibition ($P<0.05$) of the rate hair follicle growth to 0.63±0.05 mm per 72 h (mean±S.E.M.); Minoxidil at this concentration had no significant effect on the rate of either [*methyl*-^{3}H]thymidine or [U-^{14}C]leucine uptake, or on the hair follicle ATP content.

The effects of growth factors and mitogens on the longer-term growth of human hair follicles in vitro

The effects of serum on the longer-term growth of hair follicles *in vitro* is shown in Fig. 6 and shows that serum has an inhibitory effect on hair follicles maintained over a 5-day period. It was observed that at 5 days hair follicles maintained in serum-free medium were still growing in a linear fashion; but that hair follicles maintained with 1% FCS were significantly inhibited ($P<0.01$) as was the growth of hair follicles maintained in 20% FCS ($P<0.01$). There was no significant difference between hair follicles maintained in 1% and 20% FCS.

The effects of growth factors and TPA are shown in Fig. 7. This figure shows that both TGF-β1 (10 ng ml^{-1}) and TPA (100 ng ml^{-1}) significantly inhibited human hair follicle growth after 5 days in culture ($P<0.01$) and ($P<0.001$), respectively, when compared to control experiments. EGF (10 ng ml^{-1}) and IGF-1 (30 ng ml^{-1}) had no significant effect on hair follicle length when compared to controls.

The effects of Minoxidil on hair follicles maintained for 5 days is shown in Fig. 8 and shows that 200 ng ml^{-1} Minoxidil appeared to stimulate hair follicle growth significantly over 5 days ($P<0.01$) when compared with controls. Minoxidil at 10 μg ml^{-1} had no significant effect on hair follicle growth; whereas 200 μg ml^{-1} Minoxidil significantly inhibited hair follicle growth ($P<0.01$).

Discussion

In this study we have demonstrated for the first time the successful maintenance and growth of human hair follicles *in vitro*. We have shown that human hair follicles isolated by microdissection and maintained free-floating show a significant increase in hair follicle length ($P<0.01$) over 4 days in culture when maintained in Williams E medium containing 1% FCS. The rate of increase in hair follicle length was 0.3 mm/day, and this rate of increase approximates to that seen in the *in vivo* scalp hair follicle (Myers and Hamilton, 1951). It was observed that this increase in hair follicle length was not associated with any loss of hair follicle architecture and, furthermore, it was observed that the increase in hair follicle length was associated with an increase in the length of the keratinised hair shaft.

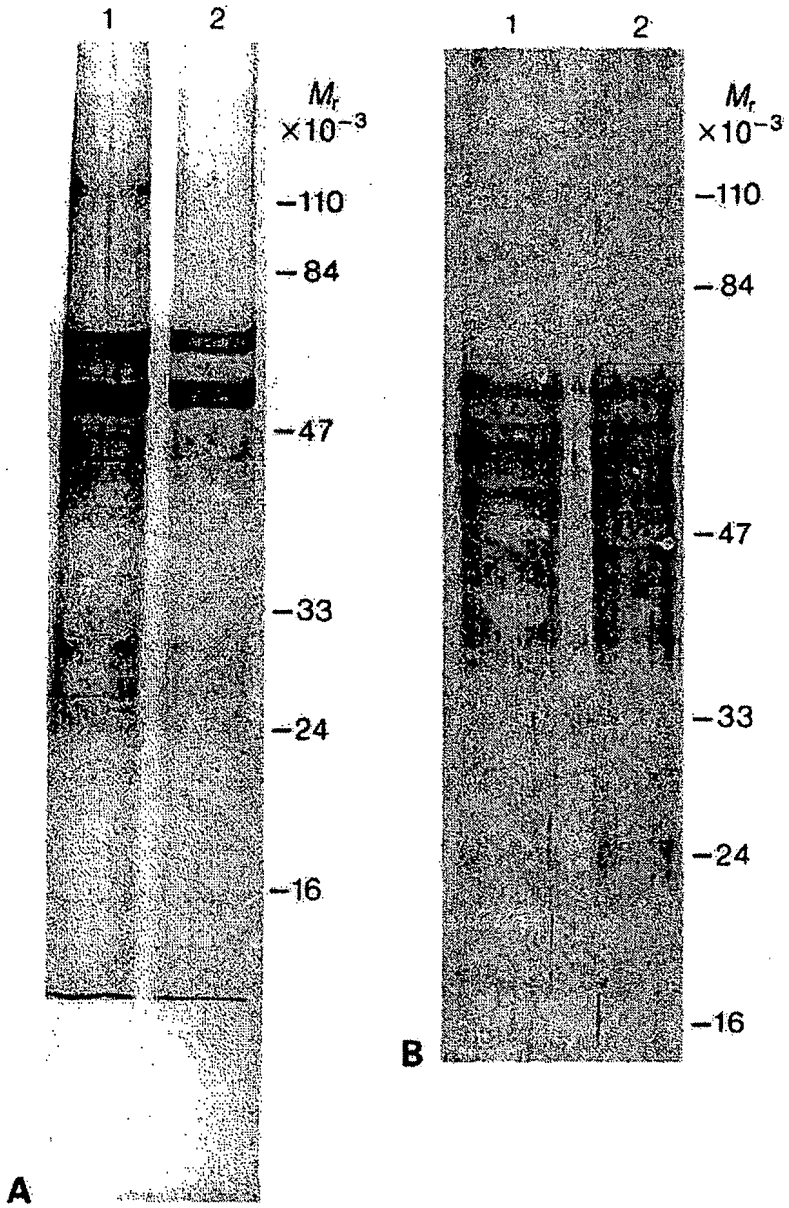

Fig. 4. (A) [^{35}S]methionine autoradiography showing the pattern of keratin synthesis in freshly isolated hair follicles (lane 1) and after 96 h maintenance (lane 2), and showing that the pattern of keratin synthesis remains unchanged in maintained hair follicles. All gels were loaded with the total keratin extract from five hair follicles.
(B) Immunoblot carried out using a broad spectrum anti-keratin antibody on freshly isolated hair follicles (lane 1) and after 96 h maintenance (lane 2). Confirming that the bands observed in A are keratins.

Further evidence to support the successful maintenance of hair follicles *in vitro* is demonstrated by [*methyl*-^{3}H]thymidine autoradiography. In freshly isolated hair follicles the majority of DNA synthesis takes place in the matrix cells of the hair follicle bulb. It was observed that in hair follicles maintained for 4 days this pattern of synthesis was maintained. These data show that *in vitro* hair follicles are able to maintain the *in vivo* pattern of DNA synthesis, and so it is reasonable to suppose that the production of a keratinised hair shaft in the maintained hair follicle occurs as a result of matrix cell division in the hair follicle bulb.

We have also observed that the pattern of keratin synthesis observed in freshly isolated hair follicles is sustained in hair follicles maintained *in vitro* for 4 days. These observations on the patterns of keratin synthesis are, however, only a preliminary study, and to characterise fully the patterns of keratin synthesis would require two-dimensional gels. However, these data confirm that the overall patterns remain unchanged and support our other observations, and also show that we are able to maintain human hair follicles *in vitro* successfully, and that they continue to produce a keratinised hair shaft.

To demonstrate further the importance of this model we have studied the effects of a number of growth-regulatory factors and mitogens on the *in vitro* rates of hair follicle elongation, [*methyl*-^{3}H]thymidine and [U-^{14}C]leucine uptake and on hair follicle viability as determined by measuring their ATP contents. The most dramatic effects were observed when the hair follicles were maintained with EGF (10 ng ml^{-1}). EGF receptors are found on rat and human hair follicles (Moore *et al.* 1981; Green *et al.* 1983), and *in vivo* experiments on mice show that EGF appears to act as a specific inhibitor of matrix cell division (Moore *et al.* 1981). In sheep, EGF acts as a depilatory by inducing a premature anagen-to-catagen transformation. As a result of this the hair fibre is weakened and this permits hand shearing (Panaretto *et al.* 1984).

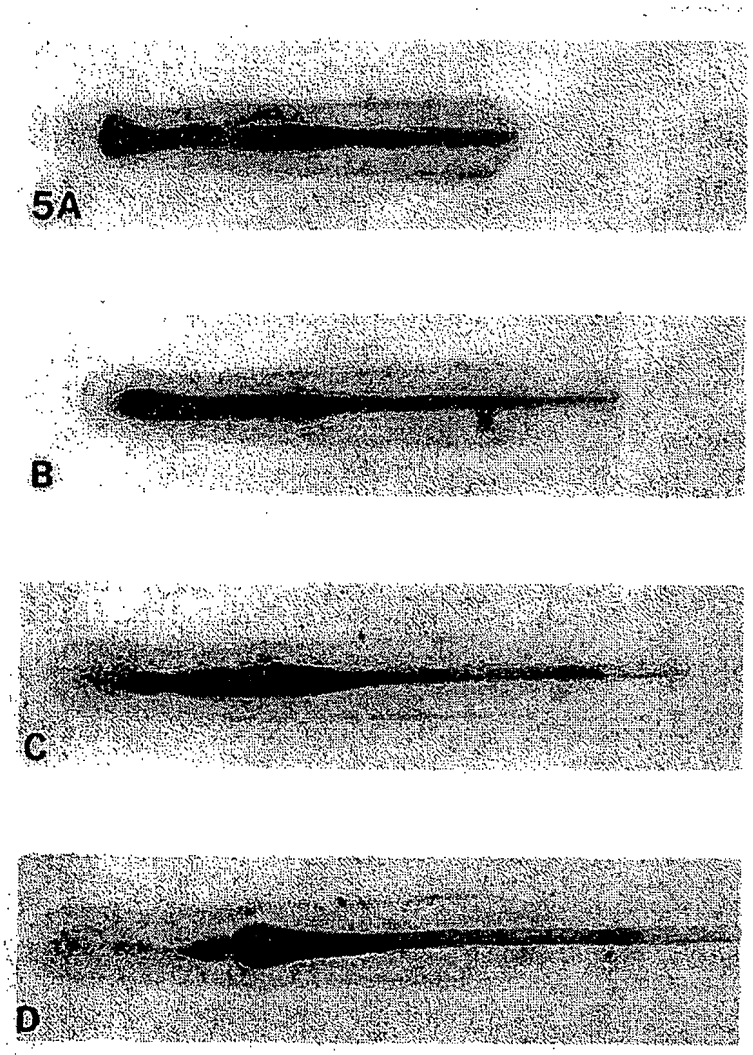

Fig. 5. Light micrograph taken under an inverted microscope showing the sequential effects of epidermal growth factor on the same hair follicle maintained in culture over a 72 h period: (A) freshly isolated hair follicle; (B) after 24 h in culture; (C) 48 h; (D) 72 h, showing the formation of a club hair-like structure, which apparently migrates upwards in the hair follicle, and resembles the *in vivo* depilatory action of EGF. The hair follicle shown in this figure was isolated from a dark-haired individual and shows a highly pigmented region adjacent to the dermal papilla, this contrasts with the hair follicle shown in Fig. 2, which was taken from a fair-haired individual and as such is not so highly pigmented. However, the EGF effect is the same in both pigmented and non-pigmented hair follicles.

In our model we have shown that isolated human hair follicles maintained with EGF show considerable morphological changes. We have found that EGF promotes the formation of a club hair-like structure, which appears to migrate upwards in the hair follicle until by day 5 it is virtually extruded from the hair-follicle. This *in vitro* depilatory effect, which appears to mimic the *in vivo* action of EGF, confirms the value of this model in hair-follicle biology, and may also point to a possible role for EGF in regulating anagen to catagen transformation during the hair growth cycle. Table 1 shows that in the presence of EGF the hair follicles remain viable as determined by both the hair follicle ATP content and [U-^{14}C]leucine uptake; however, as expected, the rates of [*methyl*-^{3}H]thymidine uptake are significantly reduced.

Immunohistochemistry has shown that TGF-β1 is present in the mammalian dermal papilla (Heine *et al.* 1987) although the biological activity of this form is not clear. Its *in vivo* function in the hair follicle is also not known; however, we have now shown that *in vitro* TGF-β1 inhibits hair growth. Table 1 shows a significant reduction in both the rates of hair follicle lengthening and [*methyl*-^{3}H]thymidine uptake in response to 10 ng ml^{-1} TGF-β1. However, this is not accompanied by the gross morphological alterations observed with EGF. The TGF-β1 effect *in vitro* is curious because, although TGF-β has been shown to be a potent inhibitor of proliferation of epithelial cells *in vitro* (Sporn *et al.* 1987), it is known that topical TPA stimulates mouse hair growth (Ogawa and Hattori, 1983; Inohara *et al.* 1988), and it has also been shown that topical TPA promotes the expression of TGF-β mRNA *in vivo* (Akhurst *et al.* 1988). However, we show in Table 1 that, *in vitro*, TPA also inhibits hair follicle growth. This is compatible with our *in vitro* TGF-β1 effect, and indicates that *in vivo* there may be further uncharacterised dermal paracrine phenomena or dose-response effects.

Dermal fibroblasts produce IGF-1 *in vitro* (Messenger, 1989); however, in our model we found no increase in hair follicle length *in vitro*, despite the significant stimulation of [*methyl*-^{3}H]thymidine uptake. However, it is possible that the insulin present in our supplemented culture medium is saturating the IGF-1 receptors, although this would not explain the observed stimulation of [*methyl*-^{3}H]-thymidine uptake.

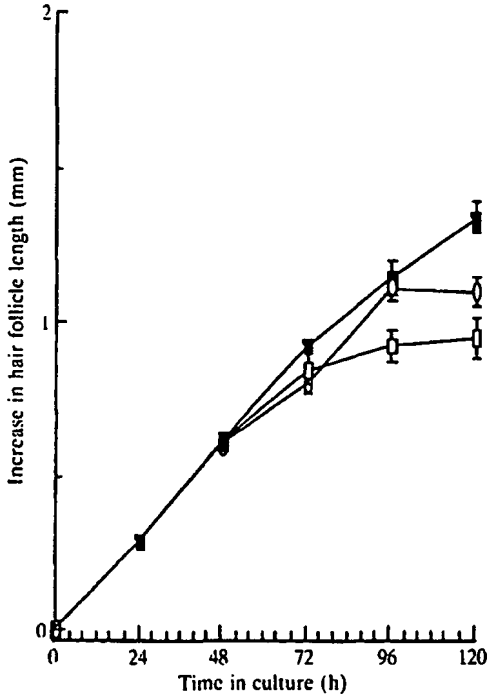

Fig. 6. Graph showing the effects of serum on isolated human hair follicles maintained *in vitro* over 5 days. Results are expressed as the mean±S.E.M. for sequential measurements made on hair follicles isolated from $n=6$ skin biopsies (6 hair follicles used from each biopsy, 36 hair follicles in total). (■) Serum-free; (○) 1% FCS; (□) 20% FCS.

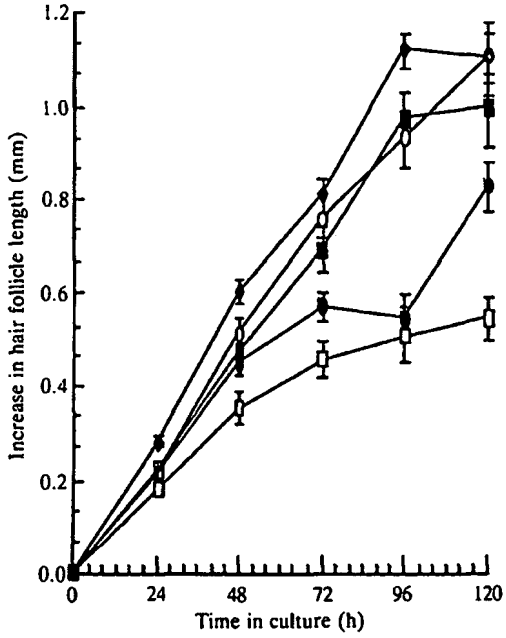

Fig. 7. Graph showing the effects of growth factors and mitogens on isolated human hair follicles maintained *in vitro* over 5 days in the presence of 1% FCS, EGF (10 ng ml^{-1}), IGF-1 (30 ng ml^{-1}),TGF-β1 (10 ng ml^{-1}) and TPA (100 μg ml^{-1}). Results are expressed as the mean±S.E.M. for sequential measurements made on hair follicles isolated from $n=6$ skin biopsies (6 hair follicles used from each biopsy, 36 hair follicles in total). (◆) 1% FCS; (○) IGF-1; (■) EGF; (●) TGF-β1; (□) TPA.

In this study we have also looked at the effect of serum on maintained human hair follicles and shown that when human hair follicles were maintained in serum-free medium they were still growing in a linear fashion after 5 days in culture whereas hair follicles maintained in tissue culture medium containing 1% FCS were significantly inhibited after 5 days in culture ($P<0.01$), as were hair follicles maintained in 20% FCS ($P<0.01$); there was no significant difference between hair follicles maintained in 1% or 20% FCS. These observations indicate that human hair follicles maintained in culture do not apparently have a requirement for serum for elongation and that serum factors may in fact be inhibitory. It is known that TGF-β is present in serum (Childs *et al.* 1982) and as we have already shown in this study TGF-β is a potent inhibitor of hair follicle growth *in vitro*. It will be interesting to see whether it is TGF-β in the serum that is inhibiting hair follicle growth or whether other inhibiting factors are present.

Minoxidil stimulates human hair growth *in vivo* (Clissold and Heel, 1987). We found, however (Table 1), that up to 72 h *in vitro* Minoxidil either had no effect, or at 200 μg ml^{-1} significantly inhibited hair growth, without effect on cell viability. Recent *in vivo* studies, however (Frienkel *et al.* 1989), indicate that Minoxidil may not increase the rate of hair growth as such, but rather it may increase the length of anagen by shortening the time that the hair follicle is in the resting stage of its growth cycle. We would, therefore, not necessarily have expected a Minoxidil stimulation of an anagen hair follicle that was already growing at a rate close to that seen *in vivo*.

However, for hair follicles maintained for 5 days in the presence of Minoxidil we observed that 200 ng ml^{-1} Minoxidil (0.95 μM) significantly stimulated hair follicle growth. This was not apparently due, however, to an actual stimulation of hair growth, it appeared that the Minoxidil was counteracting the serum-induced cessation of hair growth at 4 days. This model, therefore, may be useful for dissecting Minoxidil's mode of action. The concentration of Minoxidil that was active in our hands contrasts with those of Buhl *et al.* (1989) and Waldon *et al.* (1989), who have reported, using a rat vibrissae culture system, that the minimum effective dose of Minoxidil is between 0.5 mM and 1.0 mM. In the human hair follicle we have found that 10 μg ml^{-1} (48 μM) Minoxidil had no significant effect on human hair follicles whereas 200 μg ml^{-1} (0.95 mM) significantly inhibited hair follicle growth. Again these observations contrast with those of Buhl *et al.* (1989), who report that only a concentration as high as 10 mM Minoxidil is inhibitory. Clearly, human hair follicles isolated by the methods described here are much more sensitive to Minoxidil.

In conclusion, we have developed an *in vitro* model for human hair growth that reproduces the *in vivo* rate of hair growth, as well as the apparent *in vivo* pattern of cell division in the hair follicle matrix cells. The importance of this model in hair follicle biology has been demonstrated by the *in vitro* effects of EGF, which mimic those seen *in vivo*, and by the growth inhibitory effects of TGF-β1. Also, the observation that hair follicles grow for a longer period of time in serum-free medium suggests that they are able to regulate their own growth, possibly by the production of relevant growth regulatory factors. This should prove

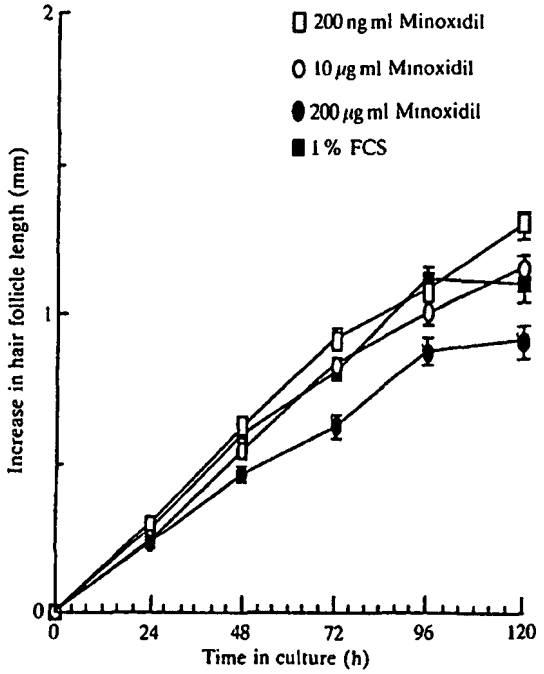

Fig. 8. Graph showing the effects of Minoxidil on isolated human hair follicles maintained *in vitro* over 5 days. Results are expressed as the mean±S.E.M. for sequential measurements made on hair follicles isolated from $n=6$ skin biopsies (6 hair follicles used from each biopsy, 36 hair follicles in total). (□) 200 ng ml^{-1} Minoxidil; (○) 10 μg ml^{-1} Minoxidil; (●) 200 μg ml^{-1} Minoxidil; (■) 1% FCS.

useful in identifying the autocrine/paracrine mechanisms that operate in the hair follicle.

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PERSONAL SKILLS

➲ Research

- Knowledge of research topics in different areas of cosmetics: skincare, haircare, make-up, sun protection
- Devising and monitoring operational research projects and managing groups of horizontal projects
- Scientific communication

➲ Management

- General Management of a Research company
- Management of Research Departments containing multi-disciplinary teams with 75 employees
- Management of technical and human resources functions
- Responsible for budgets of several thousand euros

➲ Marketing

- Overall knowledge of the cosmetics markets: segmentation, growth, trends
- Mastery of development processes for new active ingredients and research/marketing relationships
- Knowledge of purchasing processes in large cosmetics Groups

PROFESSIONAL EXPERIENCE

MANAGING DIRECTOR of the European Institute of Cellular Biology

*Since 01/01/2004, Managing Director of a biotechnology company with 10 members of staff, whose business is the Research and Development of peptide ingredients for cosmetics and dermatology.

MH CONSULTANT, technical innovation consultancy firm

*Since 01/10/1999: Director of MH Consultant, a technical innovation consultancy firm in the areas of cosmetics, dermatology and fine chemistry

- Setting up a network of experts
- Working with:
  - *the fine chemistry sector, to produce new raw materials: Rhodia, Colética etc.
  - *the cosmetics sector, to devise new concepts and research active ingredients and formula systems: YSL, LVMH, Clarins
  - *the dermatology sector, in the area of sun protection: Pierre Fabre Dermocosmétique

*From 01/10/98 to 01/10/99: Exclusive scientific consultant to the L'Oréal Advanced Research department

L'OREAL- RESEARCH

*16/10/96 to 18/09/98: Director of Make-up Applied Research Department

- Founding of a new AR department dedicated to Make-up
- Defining its organisation and putting in place a recruitment drive to increase the size of the department from 12 to 40
- Organising a project operating method

*01/06/94 to 16/10/96: Director of Chemistry Department

- Regrouping all the similar units into one standard Department with 75 staff
- Setting up a new multi-disciplinary organisation to focus on rapid integration of marketing and development needs

*18/10/89 to 31/05/94: Head of Section, Chemistry

- Responsible for three research units with 30 researchers

*03/01/77 to 18/10/89: Research engineer, then Head of a research unit

- Responsible for a unit of 10 people, reporting to the Advanced Research Manager
- Responsible for 2 projects: research into anti-ageing molecules and anti-hair loss molecules

MAIN ACHIEVEMENTS

MANAGEMENT and MARKETING

- Preparing development and distribution contracts
- Managing development contracts between companies supplying raw materials and cosmetics companies

- Management of Research Departments
- Founding a new Advanced Research Department
- Scientific collaboration with a number of public research centres – the CNRS (French National Centre for Scientific Research) and Universities – and industry – suppliers of raw materials

OPERATIONAL RESEARCH

- As Director of Make-up AR:
  *initialisation of new concepts
  *transferring technologies to the make-up sector from other sectors such as the food processing industry

- As Director of the Chemistry Department:
  *"new colour families" project to improve the durability of hair colour
  *"ceramides" project leading to the industrial synthesis of the "R" ceramide, essential progress in terms of hair protection
  *"new UV/A sun filter" project, leading to the "Mexoryl XL" derivative, an absorbent lipophilic sun filter in UV/B and A

- As a Researcher and Head of unit:
  *coordinator of the anti-hair loss project and responsible for the programme to synthesise new anti-hair loss derivatives, leading to a new active ingredient used in several ranges: Aminexil

*responsible for the programme to synthesise anti-ageing molecules, leading to the new derivative lipophilic hydroxy acid formulated in various Vichy, Lancôme and La Roche Posay commercial products.

PUBLICATIONS and PATENTS

- Oral and written scientific publications for numerous launches
- 30 scientific publications
- Patents: 3 patents filed with the company IEB
  - 34 patents filed personally with L'Oréal
  - over 100 patents filed under my responsibility

EDUCATION

- 1998: International Management seminar at CEDEP (European Centre for Continuous Education)
- 1996: Recruitment Interviewing seminar (SHL France)
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- 1992: Management seminar (RITME International)
- 1991: communications seminars (KS Communications)

- 1971-1976: Preparation of a doctorate, Université-Paris VI at CERCOA/CNRS (French National Centre for Scientific Research) "synthesis and pharmacological research in the area of analgesics"
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- French Polymers Group
- French Society of Cosmetology
- French Polyphenols Group
- French Association for the promotion of economic intelligence